INTRODUCTION

Among the many mRNAs synthesized within a cell, some have the unusual property of being localized to spatially restricted domains of the cytoplasm. Not surprisingly, in most cases a clear rationale is apparent for localization of those mRNAs. For example, actin mRNA is preferentially localized to the growing margins of cultured fibroblasts, where demand for the encoded proteins is greatest (Lawrence and Singer, 1986). A group of examples related to one another are the Drosophila ‘pair-rule’ segmentation genes, whose transcripts are localized to the apical sides of the syncitial nuclei in blastoderm stage embryos (Ingham et al., 1985; Weir and Kornberg, 1985; Macdonald et al., 1986; Gergen and Butler, 1988); proteins encoded by these transcripts act in defining rather precise spatial domains ([reviewed by Ingham and Martinez-Arias (1992)] and apical localization is thought to help prevent the proteins from diffusing throughout the syncitium (Davis and Ish-Horowicz, 1991). In Xenopus, a variety of maternal mRNAs are localized to either the animal or vegetal hemispheres of the oocyte (Rebagliati et al., 1985; King and Barklis, 1985). A set of maternal mRNAs found in the Drosophila ovary provide what are perhaps the most elaborate examples of mRNA localization characterized thus far. Briefly, transcripts of a small group of genes are shuttled between interconnected cells, starting in a group of support cells, the nurse cells, and moving into the developing oocyte. Once in the oocyte, a few of these transcripts are further localized; the best characterized examples are bicoid (bcd) mRNA, which is positioned at the anterior pole of the oocyte (Berleth et al., 1988; St. Johnston et al., 1989), and oskar (osk) mRNA, which becomes highly concentrated at the posterior pole (Kim-Ha et al., 1991; Ephrussi et al., 1991). In each case localization is a critical prerequisite to the subsequent deployment of a body patterning morphogen from the respective pole of the embryo ([reviewed by St. Johnston and Nüsslein-Volhard (1992)].

Although many localized mRNAs have now been characterized, it is not yet understood how localization is achieved. In all examples for which a localization signal has been identified, including the Drosophila transcripts of bcd (Macdonald and Struhl, 1988), fsK10 (Cheung et al., 1992), oskar (J. Kim-Ha, P. Webster, J. L. S. and P. M. M., unpublished data), even-skipped, hairy (Davis and Ish-Horowitz, 1991) and nos (Gavis and Lehmann, 1992), Vg1 from Xenopus (Mowry and Melton, 1992) and MAP2 from rat cells (Garner et al., 1988), the process is mediated by regulatory elements present within the transcript itself. Insights about mechanisms may be expected from analysis of these control elements, which are presumably binding sites for localization proteins. However, almost all such elements are at present imprecisely defined, usually as large regions. Moreover, likely binding sites for localization proteins have not been identified.

Similarly, very little is known about the proteins that mediate and control mRNA localization. On a broad scale, cytoskeletal elements are known to be required for the localization of at least the Vg1, bcd and actin mRNAs.
(Yisraeli et al., 1990; Pokrywka and Stephenson, 1991; Sundell and Singer, 1991). Microtubule and microfilament inhibitors have been shown to disrupt and therefore define, different stages in localization of Vg1 mRNA (Yisraeli et al., 1990). Similarly, microtubules are necessary for localization of bcd mRNA (Pokrywka and Stephenson, 1991) and the pattern in which microtubules are organized within the Drosophila egg chamber hints of a role in the localization of other mRNAs (Theurkauf et al., 1992). However, only a few factors are known to be more specifically involved in mRNA localization. In Drosophila, several genes required for localization of maternal mRNAs have emerged from genetic screens for mutants. For example, the exuperantia (exu), swallow (sww) and staufen (sta) genes are all required for localizing bcd mRNA to the anterior pole of the egg and later the embryo (St. Johnston et al., 1989; Stephenson et al., 1988). Each mutant alters bcd mRNA distribution at a particular point during localization, thus defining steps in the process. All three genes have been cloned and sequenced (Stephenson et al., 1988; Macdonald et al., 1991; Marcey et al., 1991; Chao et al., 1991; St. Johnston et al., 1991), but only sta has been associated with a biochemical activity. The sta protein includes multiple copies of a domain shown to mediate binding to double-stranded RNA (St. Johnston et al., 1992) and so is expected to bind directly to bcd mRNA. However, sta can also act only late in the bcd localization pathway (St. Johnston et al., 1989) and some other protein must recognize and bind to bcd mRNA earlier. This protein may not be encoded by either exu or sww and it is likely that other factors required for bcd localization have not been identified by genetic analyses.

One general approach to learning more about mRNA localization is to define precisely the cis-acting localization elements, as their makeup and organization may reveal something about the logic of the process. In addition, it should be possible to use individual regulatory elements as probes to isolate localization proteins with which they interact. Taking this approach one step further, we might expect to find peripheral cytoskeletal proteins or molecular motors among the cellular factors that interact with specific RNA-binding proteins.

Here we describe the initial steps of an in-depth analysis of the bcd mRNA localization signal. In previous work using hybrid mRNAs, a signal necessary and sufficient to direct mRNA localization to the anterior of embryos was mapped to a 0.63 kb region contained entirely within the 3′ untranslated region (3′ UTR) of the bcd mRNA (Macdonald and Struhl, 1988). Initial attempts to define this signal further met with limited success; deletion of 120-150 nucleotides (nt) from either end of the region eliminated localization. We now take a different approach, examining the organization of the bcd localization signal in its normal context, the bcd mRNA. In experiments described here, we survey the bcd mRNA for functional localization elements, using small deletions that collectively remove most of the 3′ UTR. These mutants define multiple domains involved in localization. We then focus on a more detailed characterization of one key regulatory element responsible for the early steps in localization.

### MATERIALS AND METHODS

#### Construction of mutant bcd genes

All constructs are based on the same general plasmid, p2001, consisting of an approximately 7.5 kb Asp718-EcoRI fragment of genomic bcd DNA inserted into a modified version of the CaSpR transformation vector. When cut with XbaI, this plasmid is divided into two fragments, one containing a part of the bcd gene including all of the UTR. Mutations were introduced into subclones of this XbaI fragment, which were then used to replace the wild-type fragment of p2001.

**Δ1-Δ21**

Two series of Bal31-generated deletion constructs were used to make the small internal deletions within the bcd 3′ UTR. In one series of constructs (5′ halves) the deletions extended from the StuI site near the 3′ end of the transcript towards the 5′ end of the gene. In the second series of constructs (3′ halves), the deletions extended from the SacII site near the end of the coding region towards the 3′ end of the gene. In both series, BanHI 8-mer linkers (CGGATCCG) were inserted adjacent to the deletion end points, which were mapped by DNA sequencing. Appropriate 5′ and 3′ halves were joined by ligation at the BanHI sites, creating the mutant XbaI fragment to replace the wild-type fragment of p2001. The endpoints of the various mutants are provided in Table 1.

**bcd genes tagged with lacZ**

For some constructs, the bcd gene was tagged by the addition of a fragment of the lacZ gene. The StuI site near the 3′ end of the bcd transcription unit was first converted to a NotI site by the addition of a linker. Then a 1.3 kb lacZ fragment (extending from about the start of the coding region to an MluI site) bounded by NotI linkers was inserted.

**Δ14 region constructs**

Four constructs were made to determine if the region deleted in

<table>
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<tr>
<th>Construct</th>
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*Sequence coordinates (Berleth et al., 1988) indicating the nucleotides deleted (inclusive). Note that in each case the deletion is accompanied by the insertion of a BanHI 8-mer.
Δ14 could provide localization activity in the absence of most of the remainder of the bcd 3′ UTR. In one, Δ21 was modified by the addition of the lacZ tag, to make Δ21+lacZ. This construct lacks almost all the the bcd 3′ UTR and acts as a negative control in the appropriate transgenic flies. In the other constructs, one or two copies of a 53 nt fragment of the Δ14 region [nt 4465-4517 of Berleth et al. (1988)], bounded at the 5′ and 3′ ends by BamHI and BgIII linkers, respectively, were inserted into the BamHI site defining the deletion of Δ21; 1× BLE1+ and 2× BLE1+ have one or two copies, respectively, in the sense orientation, while 2× BLE1− has two copies in the antisense orientation. All three constructs were further modified by the addition of the lacZ tag.

**Fly strains**
The bcdΔ, exuΔ and w1118 mutants and balancer chromosomes are described in Lindsley and Zimm (1992).

**Generation and characterization of transgenic fly strains**
Transgenic fly strains were generated by P-element-mediated transfection (Rubin and Spradling, 1982; Spradling and Rubin, 1982) using w1118 embryos as recipients for microinjection with p2A-3 as the helper plasmid. Some transgenic lines with lacZ tags were used without further characterization of the sites of transgene insertion. For constructs to be tested for rescue of bcd function, standard segregation tests were used to identify chromosomes carrying transgenes. Insertions on the second chromosome were crossed into a bcd mutant background to give stocks of the following generic genotype: P[wΔ, bcd(altered)]CyO; bcdΔ/TM2. These stocks were used to generate flies for analysis.

Flies homozygous for bcdΔ and either heterozygous (1×) or homozygous (2×) for a bcd transgene were collected to measure rescue of the bcd mutant maternal effect lethality. In the rescue assay presented in Fig. 1, 1× or 2× flies were placed into a vial in which eggs were laid over a period of several days. If adult flies emerged from the vial after further incubation, rescue was scored as positive. Hatch rate analyses were also performed to provide a more quantitative assay (these data are not presented here but are available on request). 1× or 2× flies were placed into cages with yeasted apple juice plates. After two days at 25°C, egg collections for hatch rate determination were begun. Eggs from a single collection (3-12 hours) were aged for 36 hours at 25°C to allow hatching. Following treatment with bleach to remove chorions, all embryos and larvae were first collected into a filtration device by washing the plate with a stream of 0.1% Triton X-100, then transferred into a microfuge tube and finally mounted in Hoyers on a microscope slide. Slides were viewed under dark-field illumination, and the hatched larvae and unhatched embryos were counted, ignoring unfertilized eggs. Typically, when bcd mRNA is not correctly localized, as in exu, sno1 and stau mutants, most or all of the embryos arrest development before hatching. Thus the hatch rate provides a measure of localization. For cuticle preparations, the embryos were devitellinized prior to mounting by shaking in a 1:1 mixture of methanol and heptane.

Some variation in both rescue and hatch rates was encountered among independent lines of flies carrying the same mutant bcd transgene (Fig. 1; data not shown). This could not be simply explained by significant differences in transcript levels; for some of the constructs all different transformant lines were tested for transcript levels (relative to the endogenous bcd gene) and found to be quite similar, with very few exceptions (data not shown). The two simplest explanations for the variability in rescue are as follows. First, very small differences in transcript levels may in fact affect rescue. If so, we expect that this effect is most pronounced when localization is impaired. Second, there may be a transcriptional component to mRNA localization. It is possible that something reminiscent of ‘gene gating’ (Blobel, 1985) is happening. Depending on the site of transgene insertion into the genome, the transcripts may be directed to exit the nucleus at a site favorable, neutral, or unfavorable for localization. Transcripts from the unfavorable sites might enter the localization pathway less efficiently than those from the other sites. Because this would be a defect in efficiency, we would again expect the effect to be most pronounced when localization is impaired.

**RESULTS**
In order to test the relative contributions to mRNA localization made by different parts of the bcd 3′ UTR, we established a series of transgenic fly strains, each carrying a bcd transgene which has a mutation in the 3′ UTR, but encodes a wild-type bcd protein (Fig. 1). Using standard genetic manipulations each transgene was introduced into a bcd− background, creating fly strains in which the only transgene inserted was the one being tested. Flies homozygous for the bcdΔ allele can be distinguished from those of the transgenes, since the bcdΔ mutation is a deletion lacking part of the sequences corresponding to the probe (Struhl et al., 1989).

In situ hybridization to whole-mount preparations of ovaries and embryos followed the method of Tautz and Pfeifle (1989) as described (Macdonald, 1992). In most cases an antisense RNA probe prepared by in vitro transcription (J. Kim-Ha, P. Webster, J. L. S. and P. M. M., unpublished data) and corresponding to the lacZ fragment was used. Two constructs (Δ21+ and 2× BLE1−) carried the lacZ fragment in the antisense orientation. For these a sense transcript was used as a probe. Most constructs of this series (all but 1× BLE1+) were also analyzed with a double-stranded DNA probe prepared by random priming, to ensure that the mRNAs were detected with similar efficiencies.

**RNA analysis**
RNAAse protection assays were used to compare levels of endogenous bcd transcripts and transgene transcripts. RNA samples were prepared as described (Macdonald et al., 1986) after dissecting 5-10 pairs of ovaries from either 1× or 2× flies that were also homozygous for the bcdΔ mutation. Radiolabeled antisense RNA probes used for the assay were generated by T7 transcription. For one probe the template was p1116, which consists of a SacI-SalI bcd restriction fragment [nt 2218-2764 of Berleth et al. (1988)] in pGEM2, cut with EcoRI (which flanks the SacI site in the poly linker). Using this probe, transcripts of the endogenous genes (the bcdΔ allele) can be distinguished from those of the transgenes, since the bcdΔ mutation is a deletion lacking part of the sequences corresponding to the probe (Struhl et al., 1989).
almost all of the 3′ UTR) reveals that Δ21 produces substantial bcd body patterning activity, indicating that translation is not severely reduced (Fig. 3A-C). For the key deletion mutants, we have confirmed that mRNA localization is indeed affected, as described below.

Mutants included in the initial survey of the bcd 3′ UTR include twenty small (~50 nt) deletion constructs, which collectively delete almost all of the 3′ UTR (Δ1-Δ20), and one large deletion construct, Δ21, which lacks most of the 3′ UTR and serves as a negative control for localization. The rescuing properties of these mutants are presented in Fig. 1 and are considered in more detail in the following sections.

**Mutants with no strong effect on bcd mRNA localization**

The majority of the small deletion mutants (13 of 20) behave like wild type in that one copy of the gene is sufficient to provide rescue of the bcd<sup>Δ</sup> phenotype (1X rescue in Fig. 1). Among the regions of the 3′ UTR included in this category is a segment found in a previous study to be necessary for localization of a lacZ<sub>bc</sub> fusion mRNA (Macdonald and Struhl, 1988). In those experiments, most of the bcd 3′ UTR was fused to the lacZ gene and shown to direct its anterior localization in embryos. Localization was lost after deletion of about 120 nt from the 5′ end of the bcd segment, a region corresponding to Δ3, Δ4, and Δ5. To facilitate comparison of the current and earlier experiments, we constructed a composite Δ3-Δ5 deletion mutant. This mutant behaves like wild-type in the bcd mutant rescue assay (Fig. 1), in marked contrast to the result of the earlier experiment. Possible explanations for these results are presented in the Discussion.

**Mutants that impair but do not prevent bcd mRNA localization**

Several of the small deletion mutants display a reduced but still significant ability to rescue the bcd<sup>Δ</sup> mutant phenotype: one copy of the mutant transgene fails to rescue, while two copies do provide rescuing activity (Fig. 1). Presumably, localization is impaired and the absolute amount of bcd activity provided at the anterior pole by a single copy of the transgene is insufficient to specify head development. This defect is overcome when the level of bcd transcripts is doubled.

The five mutants that impair localization lack sequences spread throughout the 3′ UTR, and it is not immediately obvious why these mutants should be more defective than others. Although a detailed analysis of these regions is...
bicoid mRNA localization elements

bicoid mRNA localization elements

Fig. 3. Mutants that disrupt bcd mRNA localization retain bcd body patterning activity. Anterior portions of cuticle preparations of embryos from mothers with the following genotypes. (A) Wild-type. (B) Homozygous for bcdΔ. (C) Homozygous for bcdΔ and homozygous for Δ21. (D) Homozygous for bcdΔ and homozygous for Δ14. The wild-type cuticle displays the normal involuted head structures and three thoracic denticle belts, followed by the anteriormost abdominal denticle belts. In embryos from bcdΔ mutant mothers, all head structures are missing and are replaced by duplicated telson structures, including the filzkorper, which appear as large white spots. In addition, the thoracic denticle belts are absent. Part of the bcdΔ phenotype is rescued by the Δ21 transgene; two thoracic denticle belts form and the head structures, while still partially absent, are no longer transformed into telson. This degree of rescue can be attributed to the bcd mRNA (and functional bcd protein) now dispersed throughout the embryo. Introduction of the Δ14 transgene into bcdΔ mutant mothers restores more anterior pattern elements. Involuted head structures are now present, although incomplete and all three thoracic denticle belts form.

Beyond the scope of this work, we did perform a limited set of experiments with several examples to confirm that mRNA localization is indeed affected. Since there are no RNA null bcd alleles, it is difficult to distinguish between the endogenous and transgene bcd mRNAs in whole-mount ovaries. Therefore, for our localization studies a lacZ tag was placed near the 3′ end of several rescue-impaired constructs (Δ7, Δ11 and Δ16) to allow detection of the transgene mRNAs by in situ hybridization.

During oogenesis, wild-type bcd transcripts first appear during stages 5-6 (stages of oogenesis are as defined by King (1970) and Mahowald and Kambysellis (1978)), and are rapidly concentrated within the oocyte. As the oocyte expands, bcd mRNA is concentrated at the cortex, becoming gradually restricted to the anterior oocyte margin, which abuts the nurse cells. By stage 8 bcd, mRNA begins to accumulate noticeably in the nurse cells and the transcripts are eventually concentrated in apical zones above the nuclei. The amount of bcd mRNA at the anterior cortex of the oocyte gradually increases until the end of stage 10B, when the contents of the nurse cells are transferred into the oocyte. During a late stage in oogenesis, bcd mRNA moves from an anterior cortical position to a more internal anterodorsal location, as seen in young embryos (Berleth et al., 1988; Stephenson et al., 1988; St. Johnston et al., 1989).

These patterns of bcd mRNA distribution are largely mimicked by a tagged but otherwise wild-type bcd+lacZ transcript (Fig. 4A; the lacZ tag of about 1.3 kb is inserted close to the polyadenylation site of the bcd mRNA). The only detectable difference is in embryos; endogenous bcd mRNA is tightly restricted to a compact anterior zone (Fig. 5G), while the tagged transgenic bcd mRNA spreads out somewhat posteriorly (Fig. 5A). Thus, all tagged versions of the bcd gene have a modest localization defect.

Localization of Δ7+lacZ transcripts is normal until stage 9 or 10A, when a notable deviation from wild type appears; Δ7+lacZ transcripts are not restricted to the anterior margin of the oocyte, but are instead spread out along the cortex, accumulating in both anterior and lateral cortical regions (Fig. 3F). This relaxation of localization persists into embryos, where Δ7+lacZ mRNA is dispersed across a broad anterior domain (Fig. 5E). This dispersion of mRNA in the embryo is frequently uniform throughout the anterior domain, although we sometimes observe a concentration gradient decreasing posteriorly.

In contrast to Δ7+lacZ, the localization patterns of both Δ11+lacZ and Δ16+lacZ more closely resemble wild type during oogenesis, although some cortical spreading occurs in stage 10A-10B oocytes (Fig. 4G; data not shown). In young embryos, there is still an anterior concentration of both transcripts, but the levels are lower and the mutant mRNAs are to some degree unlocalized and spread uniformly throughout the embryo (Fig. 5F; data not shown). Overall, localization appears to be less efficient than for wild-type.

An essential region of the bcd 3′ UTR defines bcd localization element 1 (BLE1)

Only Δ21 and Δ14 of the collection of bcd 3′ UTR deletion mutants fail to rescue the bcd− phenotype, producing completely inviable embryos. Δ21 deletes most of the 3′ UTR and serves as a negative control for localization. Δ14, in contrast, is a small deletion removing only 50 nt of the transcript (Fig. 1). We refer to the cis-regulatory site, suggested by Δ14 and confirmed by additional experiments described below, as bcd localization element 1 (BLE1).

Transgenic flies carrying a tagged derivative of Δ14, Δ14+lacZ, were generated to monitor directly the mutant
Fig. 4. Distribution of lacZ-tagged transgene mRNAs in whole-mont ovary preparations. Each panel shows egg chambers increasing in age from left to right. In all cases, the egg chambers are oriented with the oocyte to the right; within the oocyte the anterior boundary is to the left. (A) bcd+lacZ. Transcripts of the transgene are localized in essentially the same pattern as for bcd mRNA. (B) Δ14+lacZ. Localization is largely defective, although traces of the transcript can be seen at the anterior margin of all late stage oocytes shown. (C) Δ14S+ lacZ. Localization resembles that of bcd+lacZ, except that the early (stage 5-6) concentration in the oocyte is greatly reduced or absent. (D) 2× BLE1−. There is no evidence of any localization of the transcript in the normal bcd pattern. (E) 2× BLE1+. Localization begins normally, with strong concentration in stage 5-6 oocytes. Later, localization to the anterior margin is initially normal but, by stage 9, there is substantial diffusion both along the cortex and into the center of the oocyte. In the late stage egg chamber (10B or 11), little or none of the transcript remains at the anterior margin of the oocyte. However, localization within the nurse cells to apical zones above the nuclei remains normal, as easily seen in the late stage egg chamber. (In our hands this pattern is somewhat variable and does not appear in all stage 10 egg chambers, independent of genotype. The only exceptions are for Δ14+ lacZ and Δ21+ lacZ transcripts and for both tagged and endogenous bcd transcripts in exu− flies, for which this pattern is never observed.) (F) Δ7+ lacZ. Localization is normal until stage 9 or 10A (variable), when the transcripts disperse along the cortex, covering the anterior third to half of the oocyte and vacating most of the anterior margin of the oocyte. In addition, there appears to be considerably less of the transcript present in the nurse cells than normal. (G) Δ11+ lacZ. Localization is normal during the early stages. In stage 10A oocytes, there is some movement of the transcript away from the anterior margin of the oocyte, although to a much lesser extent than seen for the Δ7+ lacZ transcripts. (H) 2× BLE1+ in exuSC egg chambers. Localization begins normally, with strong concentration in stage 5-6 oocytes. Transcript distribution within the oocyte is initially much the same as for 2× BLE1+ in a wild-type background (see E). Localization at the anterior margin is initially normal, but by stage 9 little or no transcript remains localized. This delocalization precedes that observed for 2× BLE1+ transcripts in wild-type oocytes. Within the exu mutant nurse cells, 2× BLE1+ transcripts are not localized to apical zones above the nuclei.
transcript distribution by in situ hybridization. Strikingly, Δ14+ lacZ transcripts are largely unlocalized. The normal early concentration in the oocyte is not detected. During stages 8-9 there is weak and inconsistent appearance of the mutant transcripts at the anterior margin of the oocyte. This pattern persists in later stages; localization is largely eliminated, with only a low level of correctly localized Δ14+ lacZ mRNA appearing in some stage 10 oocytes (Fig. 4B) and young embryos (Fig. 5B). The Δ14 phenotype is further confirmed by the cuticular pattern of embryos from bcd⁶ flies carrying two copies of the Δ14 transgene; anterior body pattern defects are similar to those of Δ21, but not quite as severe (Fig. 3C and D).

Mutants Δ13 and Δ15, whose deletion endpoints overlap with the segment deleted in Δ14, both successfully rescue the bcd⁻ phenotype (Fig. 1). Since Δ14 fails to rescue, it was possible that the region uniquely deleted in Δ14, an 11 nt segment, was essential for localization and hence rescue. To address this possibility, a smaller deletion mutant was constructed in two forms, Δ14S and Δ14S+ lacZ (Fig. 6), to be used in the different localization assays. Δ14S rescues the bcd⁻ phenotype; either one or two copies provide rescue (Fig. 1). Similarly, in situ hybridization to ovaries carrying Δ14S+ lacZ demonstrates that the mRNA is in large part localized correctly in oogenesis; although the early concentration in stage 5-6 oocytes is weak or not detected, all later stages of localization appear normal (Fig. 4C). Embryos show generally tight anterior localization, with some spreading of Δ14S+ lacZ mRNA away from the anterior pole (Fig. 5C).

**BLE1 directs early steps in bcd localization**

To confirm that the Δ14 mutant deletes a localization element, we asked if BLE1 itself confers localization properties. BLE1 was added to Δ21, the mutant that lacks almost all of the 3'UTR and is completely defective in mRNA localization (Fig. 1). Four constructs were tested. Δ21+ lacZ is a...
again monitored by in situ hybridization to ovaries. The BLE1+ and 2Δ14+ lacZ mRNAs are sufficient for the early steps. Our experiments do not reveal whether BLE1 plays an active role in the later stages of localization, or if the early steps are simply a prerequisite to those that follow. Our experiments do not reveal whether BLE1 plays an active role in the later stages of localization, or if the early steps are simply a prerequisite to those that follow.

Of the genes known to be required for localization of bcd mRNA, exu appears to act earliest (St. Johnston et al., 1989) and may therefore be required for some of the steps dependent on BLE1. We examined the distribution of 2× BLE1+ mRNA in flies homozygous for the exuSC mutation, a null allele (Macdonald et al., 1991). The early concentration of 2× BLE1+ transcripts within stages 5-7 oocytes is not noticeably altered by the exu mutation (Fig. 4H), as for wild-type bcd mRNA. During stages 7-8, transcripts within the nurse cells undergo the normal shift to the anterior (BamHI linker). Occasionally, localized mRNA is absent in most stage 9 egg chambers from exu mutant ovaries, yet is present in almost all similarly staged wild-type egg chambers. In nurse cells of exu mutant ovaries, 2× BLE1+ transcripts never adopt the normal apical localization above the nurse cell nuclei. Thus, the absence of exu activity produces different effects in the different cells: a gradual loss of localization within the oocyte and a complete failure of localization in the nurse cells.

**DISCUSSION**

Positioning of mRNAs to specific subcellular locations is now recognized as a widespread phenomenon [reviewed by Gottlieb (1990)]. However, our understanding of the underlying mechanisms remains rudimentary; the cis-acting elements that mediate localization are at best incompletely defined, and trans-acting factors that recognize specific mRNAs and direct their localization have yet to be identified or isolated. Here we describe experiments that address the organization and composition of the bcd mRNA localization signal, examining the properties of a collection of small deletion mutants covering the region previously shown to contain the signal. The results of these experiments are consistent with the idea that BLE1 consists of two or more binding sites for 

Fig. 6. Definition of bcd localization element 1 (BLE1). A portion of the bcd 3′ UTR RNA sequence is presented in the top line [nucleotides 4454-4530 of Berleth et al. (1988)]. The 53-nucleotide region defined as BLE1 is overlaid with an arrow. Underneath the wild-type sequence are the same regions from mutants Δ13, Δ14, Δ15 and Δ14S, with the deletions indicated by dashes. Although not shown, all of the mutants include the sequence CGGAUCCG at the site of the deletion, corresponding to a BamHI linker.

**BLE1 directs early steps in bcd mRNA localization**

Deletion mutant Δ14 defines a region required for normal bcd mRNA localization which we call BLE1. Failure of the Δ14 mutant to localize correctly its mRNA has been demonstrated by a rescue assay and by following the distribution of a tagged version of the mRNA during oogenesis and embryogenesis. We find that localization of the mutant mRNA is largely but not completely eliminated, beginning at the earliest known stage of the process. Notably, mutants Δ13, Δ14S and Δ15 collectively delete the entire Δ14 region and fail to localize correctly its mRNA. This redundancy within the Δ14 region can be most readily explained by positing that BLE1 consists of two or more binding sites for a localization factor (or factors). Too few sites remain in Δ14 to support localization, while Δ13, Δ15 and Δ14S retain enough sites for localization to be sufficient for rescue. Since Δ14 retains a very low level of mRNA localization, there may be more binding sites for the same factor outside of BLE1, or there may be redundancy in the localization mechanisms.

Two tandem copies of BLE1 are sufficient to direct the early localization stages of a bcd mRNA lacking most of its 3′ UTR. In contrast, similar transcripts with only one copy of BLE1 or with two antisense copies of BLE1 do not direct any stage in localization. The antisense result is exactly as would be expected for an RNA regulatory element, but the failure of a single sense copy of BLE1 to support any localization is more difficult to interpret. At least two explanations are possible. First, this result could be viewed as supporting the notion that a factor that interacts with BLE1 also binds to sites elsewhere within the bcd 3′ UTR, which are absent in these constructs. Alternatively, if BLE1 is only active when folded correctly, the presence of two copies...
mRNA distribution, in which the transcripts are restricted to the anterior third of the embryo, but are fairly evenly dispersed over much of that region. The defect can be traced back to stage 10A in oogenesis, when the transcripts spread out along the lateral cortex of the oocyte. Curiously, the Δ7 pattern in the oocyte is similar to the distribution of bcd mRNA in sww ovaries, yet Δ7 retains more anterior localization in embryos than is observed for bcd mRNA in the sww mutants. The distribution of sww protein in the egg chamber does not suggest that it interacts directly with bcd mRNA; rather, sww protein is concentrated in the basal region of the nurse cells and is not restricted to the anterior margin within the oocyte (J. Hegde and E. Stephenson, personal communication). Because the onset of the Δ7 defect is prior to the stage when sww mutants deviate from the wild-type bcd pattern, the Δ7 region seems unlikely to act as a binding site for sww protein. Indeed, we do not yet know if the Δ7 element is itself a binding site, or if it plays a different role, such as stabilizing the proposed complex formed between BLE1 and a localization protein. A comparison of the deletions of Δ6, Δ7 and Δ8 does not immediately suggest what feature is lacking in Δ7 but retained in Δ6 and Δ8 (which together remove all but 5 nt of the Δ7 deletion). Additional mutants, that remove larger segments of the localization signal, may provide more satisfying answers.

Other mutants impair localization. Although we have only begun to explore these effects, we do have preliminary results with some of the other impaired mutants. The mRNA distributions of Δ11 and Δ16 could be simply explained as reductions in localization efficiency; some transcripts are localized normally in a compact anterior zone while the remaining transcripts are spread out over the entire embryo. How might a deletion mutant decrease efficiency of localization? One possible explanation centers on the issue of secondary structure. We previously argued that a potential stereotypical secondary structure which was conserved among various Drosophila species might act to ensure that critical binding sites in the localization signal were efficiently presented for interactions with localization factors [Macdonald, 1990; see also Seeger and Kaufman (1990)]. If so, then deletions that do not remove a binding site for localization factors might still have a modest effect on localization by influencing the folding of the mRNA.

Additional localization elements have been suggested in other studies. We previously found that when a 0.63 kb region of the bcd 3′ UTR was used to localize a heterologous mRNA, removal of the terminal 120-150 nt from the bcd region eliminated localization (Macdonald and Struhl, 1988). We recreated one of those deletions here (Δ3-Δ5) and found it to have no effect on rescue of the bcd+ phenotype. There are two likely explanations for the difference and both rest on the fact that the Δ3-Δ5 mutant exists in a much different context, that of an otherwise wild-type bcd gene. First, a redundant localization signal may be present in the Δ3-Δ5 deletion, with the other copy located outside of the 0.63 kb segment of bcd mRNA used in the first experiments. Second, the Δ3-Δ5 deletion may affect the localization signal by altering secondary structure. When the Δ3-Δ5 deletion is introduced into an intact bcd mRNA, the accompanying alterations in secondary structure are tolerable.
However, because the isolated 0.63 kb portion of \textit{bcd} may already be destabilized by its unusual context, the further removal of the A3–A5 region could have a major effect on RNA folding, thus disrupting mRNA localization.

Another \textit{bcd} localization element has been identified by Gottlieb (1992). Using an assay in which RNAs are tested for their ability to be retained at the anterior site of injection into early embryos, she found that deletion of a short segment near the beginning of the 3′ UTR caused partial mislocalization. This same segment is absent in our mutants Δ1 and Δ2, neither of which shows any reduction in ability to rescue the \textit{bcd}\textsuperscript{Δ} mutant phenotype. We have not made lacZ-tagged versions of these mutants and therefore cannot say if \textit{bcd} mRNA localization is slightly impaired.

**Strategies for mRNA localization**

Localization of mRNAs could in principle be directed by a single RNA recognition event; the mRNA is bound by a localization factor and shuttled from step to step by interactions tagging versions of these mutants and therefore cannot support by a grant from the National Institutes of Health already be destabilized by its unusual context, the further localizations of the factor with other components of the machinery. Our results argue against such a simple model and instead provide evidence in favor of a modular organization of localization signals. Specifically, we have identified at least two different localization elements in the \textit{bcd} mRNA localization signal, each acting at different steps in the process. Thus these same elements could be used in different combinations with other elements to direct the localization of mRNAs with ultimately different destinations within the oocyte. It is, however, not yet clear if the different elements act in an additive manner, where the complex formed by one RNA-protein (or RNA-RNA) interaction is required for subsequent interactions, or if the different localization elements can truly act independently of one another. Multiple localization elements have also recently been found in the 3′ UTR of the \textit{oskar} mRNA, with different elements mediating different steps in its localization to the posterior pole of the oocyte (J. Kim-Ha, P. Webster, J. L. S. and P. M. M., unpublished data). Thus localization signals consisting of multiple different elements may be common, at least for mRNAs displaying complex movements.

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